JOURNAL OF VIROLOGY, June 2006, p. 5686–5696 Vol. 80, No. 12
0022-538X/06/$08.00+0 doi:10.1128/JVI.02739-05

Sindbis Virus Nonstructural Protein nsP2 Is Cytotoxic and Inhibits Cellular Transcription

Natalia Garmashova,[1] Rodion Gorchakov,[1] Elena Frolova,[1,2] and Ilya Frolov[1]*

Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas 77555-1019,[1] and Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas 77555-1072[2]

Received 30 December 2005/Accepted 21 March 2006

Replication of alphaviruses in vertebrate cells strongly affects cell physiology and ultimately leads to development of a cytopathic effect (CPE) and cell death. Sindbis virus (SIN) replication causes major changes in cellular macromolecular synthesis, in which the strong downregulation of transcription of cellular mRNAs and rRNAs plays a critical role. SIN nonstructural protein nsP2 was previously proposed as one of the main regulators of virus-host cell interactions, because point mutations in the carboxy-terminal part of nsP2 could make SIN and other alphaviruses and replicons less cytopathic and capable of persisting in some vertebrate cell lines. These mutants were incapable of inhibiting transcription and downregulating a viral stress-induced cell response. In the present work, we demonstrate that (i) SIN nsP2 is critically involved in CPE development, not only during the replication of SIN-specific RNAs, but also when this protein is expressed alone from different expression cassettes; (ii) the cytotoxic effect of SIN nsP2 appears to be at least partially determined by its ability to cause transcriptional shutoff; (iii) these functions of SIN nsP2 are determined by the integrity of the carboxy-terminal peptide of this protein located outside its helicase and protease domains, rather than by its protease activity; and (iv) the cytotoxic activity of SIN nsP2 depends on the presence of this protein in a free form, and alterations in P123 processing abolish the ability of nsP2 to cause CPE.

The alphavirus genus of the *Togaviridae* family contains a number of human and animal pathogens. Nearly 30 members of the genus are widely distributed on all continents. They are transmitted by mosquitoes to vertebrates that serve as amplifying hosts (17, 20, 38). In infected vertebrates, some of the alphaviruses cause an acute disease, often characterized by high-titer viremia or host death (16, 18). Accordingly, these viruses exhibit a highly cytopathic phenotype in cell cultures of mammalian and avian origin (38).

Sindbis virus (SIN) is a prototype member of the genus and one of the least pathogenic alphaviruses. SIN is widely used in experimental research, since it can infect a wide variety of commonly used vertebrate cell lines, where it replicates to high titers approaching 10^{10} PFU/ml. Moreover, SIN replication leads to the rapid development of a cytopathic effect (CPE) and cell death within 24 to 48 h postinfection (10). The SIN genome is a single-stranded RNA of almost 11.5 kb which has a positive polarity (37) and contains a 5′ methylguanylate cap and a 3′ polyadenylate tail. After release from the nucleocapsids (42, 43), the genome is translated into the viral nonstructural proteins nsP1 to nsP4, which are encoded by the 5′ two-thirds of the genome. Together with host factors these proteins form the replicative enzyme complex (RC) required for viral genome replication and transcription of the subgenomic RNA (38). The latter RNA is encoded by the 3′ one-third of the genome and translated into the structural proteins that are components of the viral particles (30). SIN replication in vertebrate cells strongly affects cell physiology (21) by causing major changes in cellular macromolecular synthesis. These changes appear to be a multicomponent event, leading to both the strong downregulation of transcription of cellular mRNAs and rRNAs and the translation of cellular RNA templates. Accumulation of viral structural proteins in the ER and/or plasma membrane is an additional component that accelerates CPE development (10). These and, probably, other events during SIN replication, which are not, as yet, defined, lead to cell death that usually correlates with apoptosis development (22). The inhibition of transcription and translation were found to be distinctly independent events (15), and their development could be differentially manipulated by creating different mutations in SIN nsP2. Point mutations in the carboxy-terminal part of nsP2 could make viruses and replicons (virus-specific RNAs capable of replication, but lacking structural genes) less cytopathic and capable of persisting in some vertebrate cell lines (7, 9, 27). These mutants were incapable of causing transcriptional and translational shutoff and of inhibiting the activation of virus stress-inducible genes (12, 34). Another point mutation that inactivated the cleavage site between nsP2 and nsP3 did not change the ability of SIN to downregulate the translation of cellular mRNAs but strongly altered the inhibition of transcription of cellular genes normally observed during SIN infection (15). Thus, SIN nsP2 was suggested as an important factor in virus-host cell interactions, playing a critical role in the modification of the intracellular environment for the needs of SIN replication. However, to date, the molecular basis of the less-profound effects of the mutants on translation and transcription, in particular, has not yet been explained. Moreover, it remains unclear whether transcriptional and translational shutoffs in the infected cells were caused directly by nsP2 or were due to the functioning of this protein in viral RCs.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1019. Phone: (409) 772-2327. Fax: (409) 772-5065. E-mail: ivfrolov@utmb.edu.

Alphavirus nsP2 was always an attractive candidate for a role as a regulator of cellular metabolism in infected cells. This protein was shown to be a nucleoside triphosphatase (31), RNA triphosphatase (39), RNA helicase (14), and a papain-like protease (19) that processes viral nonstructural proteins. In addition, nsP2 not only was found in the replicative complexes but also was distributed in the cytoplasm and nuclei of the infected cells (15, 26, 32, 33). These data suggest that this protein also functions in processes other than the replication and transcription of virus-specific RNAs.

In the present study, we further investigated nsP2's role in the control of cellular macromolecular synthesis caused by SIN infection. Using different expression systems, we demonstrated that nsP2 itself is capable of downregulating cellular transcription and CPE development. Expression of wt nsP2 derived from the SIN genome stops cell growth, inhibits RNA transcription, and, eventually, leads to cell death. This effect depends more on the mutations in its carboxy-terminal domain than on the nsP2-associated protease activity.

MATERIALS AND METHODS

Cell cultures. BHK-21 cells were kindly provided by Paul Olivo (Washington University, St. Louis, MO). Cells were maintained at 37°C in alpha minimum essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS) and vitamins.

Plasmid constructs. Plasmids encoding replicons of Venezuelan equine encephalitis virus (VEErep) pVEErep/GFP/Pac and pVEErepL/GFP/Pac were described elsewhere (28). The replacement of the green fluorescent protein (GFP) sequence by different variants of SIN nsP2 fused in frame with a ubiquitin (Ubi) sequence was performed by standard cloning techniques. Mutagenesis of C_{481} and P_{726} codons in SIN nsP2 expressed from VEE replicons was performed using PCR, followed by cloning and sequencing of the amplified fragment. The expression cassettes with chicken β-acting promoter were designed in the pTriEx1 backbone (Novagen). They encoded the Ubi gene fused with nsP2, either having no mutations (p2) or with C_{481}→S or P_{726}→L mutations (p2m and p2L, respectively). p1234 encoded the nucleotide sequence of the entire P1234 precursor of SIN nsPs. The nsP3-coding sequence of this plasmid contained an in-frame insertion of GFP between amino acids 389 and 390 that was shown to have no detectable effect on SIN RC functioning and virus-host cell interactions (11) but allowed us to visualize the site and expression level of SIN nsP3. p12m34 differed from p1234 by having a mutation of C_{481}→S in the nsP2 gene and an Ubi sequence in the junction between nsP3 and nsP4 genes to preserve the proper cleavage of the nsP4 (see Fig. 7A for details). P1*2*34 also contained an Ubi sequence between nsP3 and nsP4, and the P123 polyprotein encoded by this cassette contained the previously described Gly→Ala mutations in the nsP1/nsP2 and nsP2/nsP3 cleavage sites that abolished the processing (35). All of the constructs that we used are presented in the corresponding figures. Sequences of all of the recombinant plasmids can be provided upon request. In the DNA transfection experiments, we also used the pLPCX plasmid (Clontech), expressing a puromycin acetyltransferase (Pac) gene from the Moloney murine leukemia virus long terminal repeat.

RNA transcriptions. Plasmids were purified by centrifugation in CsCl gradients. Before being subjected to a transcription reaction, plasmids were linearized by using the MluI restriction site located downstream of the poly(A) sequence of VEE replicons. RNAs were synthesized by SP6 RNA polymerase in the presence of a cap analog by using previously described conditions (29). The yield and integrity of transcripts were analyzed by gel electrophoresis under nondenaturing conditions. Transcription reactions were used for electroporation without additional purification.

Analysis of nsP2 cytotoxicity. BHK-21 cells were electroporated by using previously described conditions (23). In all of the experiments, 5 μg of the in vitro-synthesized RNAs was used per electroporation of 5×10^6 cells. Next, cells were seeded into six-well Costar plates (one-tenth of the electroporated cells per well) to analyze cell proliferation (if transfected cells were capable of growing) or viability (if the transfected cells were dying because of the toxicity of the expressed proteins). Puromycin selection (10 μg/ml) was performed between 6 and 48 h posttransfection. Cells were then incubated in puromycin-free medium, and viable cells were counted at the times indicated in the figures. In parallel, to evaluate the ability of replicons to persistently replicate, different dilutions of the electroporated cells were seeded into 100-mm tissue culture dishes. At 6 h posttransfection, puromycin was added to the medium to a concentration of 10 μg/ml. Colonies of puromycin-resistant (Pur^r) cells were stained with crystal violet at days 4 to 9 posttransfection, depending on their growth rates. The results are presented in the figures in CFU per microgram of RNA used for transfection.

Analysis of replication and transcription of replicon-specific RNAs. BHK-21 cells were electroporated by 5 μg of the in vitro-synthesized RNAs, and one-sixth of the cells were seeded into 35-mm culture dishes. At 15 h postelectroporation, the replicon-specific RNAs were labeled in αMEM supplemented with 10% FBS, 1 μg of dactinomycin (ActD)/ml and 20 μCi of [^{3}H]uridine/ml for 5 h. Alternatively, RNAs were labeled at 15 to 19 h posttransfection in phosphate-free MEM, supplemented with 0.1% FBS, 1 μg of ActD/ml, and 100 μCi of [^{32}P]phosphoric acid/ml. RNAs were isolated from the cells by using TRIzol reagent as recommended by the manufacturer (Invitrogen), denatured with glyoxal in dimethyl sulfoxide, and analyzed by agarose gel electrophoresis as previously described (5).

Analysis of cellular transcription. BHK-21 cells were electroporated by 5 μg of the in vitro-synthesized RNAs, and one-sixth of the cells were seeded into 35-mm culture dishes. At 6 h posttransfection, puromycin was added to the medium to a concentration of 10 μg/ml. At 15 h postelectroporation, the cellular RNAs were labeled for 5 h in the complete αMEM supplemented with 10% FBS and 20 μCi [^{3}H]uridine/ml and containing no ActD. RNA isolation and analysis by agarose gel electrophoresis were performed as described above. For quantitative analysis, the RNA bands were excised from the 2,5-diphenyloxazole (PPO)-impregnated gels, and the radioactivity was measured by liquid scintillation counting.

Analysis of apoptosis. For detection of apoptosis, BHK-21 cells were electroporated by 5 μg of the in vitro-synthesized RNAs. Next, 200 μl from 10-ml samples of electroporated cells was seeded directly onto the glass chamber slides (Nunc). Puromycin selection (10 μg/ml) was started at 6 h and, at 48 h posttransfection, cells were fixed with 1% formaldehyde in phosphate-buffered saline, permeabilized with 0.5% Triton X-100, and stained with mouse anti-phospho-histone H2A.X antibodies (Upstate) and goat anti-mouse immunoglobulin G (IgG) Alexa Fluor 546-labeled secondary antibodies (Molecular Probes). In addition, cell nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole; Molecular Probes). Staining was evaluated on an inverted UV microscope.

Analysis of SIN nsP2 accumulation. BHK-21 cells were electroporated by using 5 μg of the in vitro-synthesized replicons' RNA under the above-described conditions. Based on the numbers of GFP-expressing cells after transfection of VEErep/GFP/Pac replicons, the average electroporation efficiency under the conditions used was described above 95%. After electroporation, cells were seeded into 12-well Costar plates (1/20 of the electroporated cells per well). At 6 h posttransfection, puromycin was added to the medium to a concentration of 10 μg/ml. At 20 h posttransfection, the cells were scraped, and equal amounts of proteins from each sample were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. After transfer, the nitrocellulose membranes were stained with 0.5% Ponceau S (Fisher) in 1% acetic acid to control the quality of the protein transfer and processed by using affinity-purified rabbit anti-nsP2 antibodies (diluted 1:1,000), generated by immunizing rabbits with purified nsP2, expressed in *Escherichia coli*. Horseradish peroxidase-conjugated, secondary donkey anti-rabbit antibodies were purchased from Santa Cruz Biotechology and used in a dilution of 1:5,000. The Western blot Luminol reagent was used according to the manufacturer's recommendations (Santa Cruz Biotechnology).

DNA transfections and analysis of cell growth. DNA transfections were performed by using FuGENE 6 transfection reagent, according to the manufacturer's instructions (Roche). Briefly, BHK-21 cells were seeded into multiple, 60-mm tissue culture dishes at a density of 8×10^4 cells/dish. After overnight incubation at 37°C, they were transfected by a mixture of 3.5 μg of SIN nsP2-encoding plasmids and 1 μg of pLPCX. After 8 h of incubation with transfection reagent, media were replaced by fresh complete media. Puromycin selection (10 μg/ml) was performed between 24 and 48 h posttransfection. In the preliminary experiments, this time was found to be sufficient for eliminating all of the untransfected, Pur^s BHK-21 cells. Media were then replaced every 24 h to increase the experiments' reproducibility. Cell counting was performed at the times indicated in the figure.

RESULTS

Expression of wt SIN nsP2 is cytopathic. In the present study, we took advantage of an expression system based on the

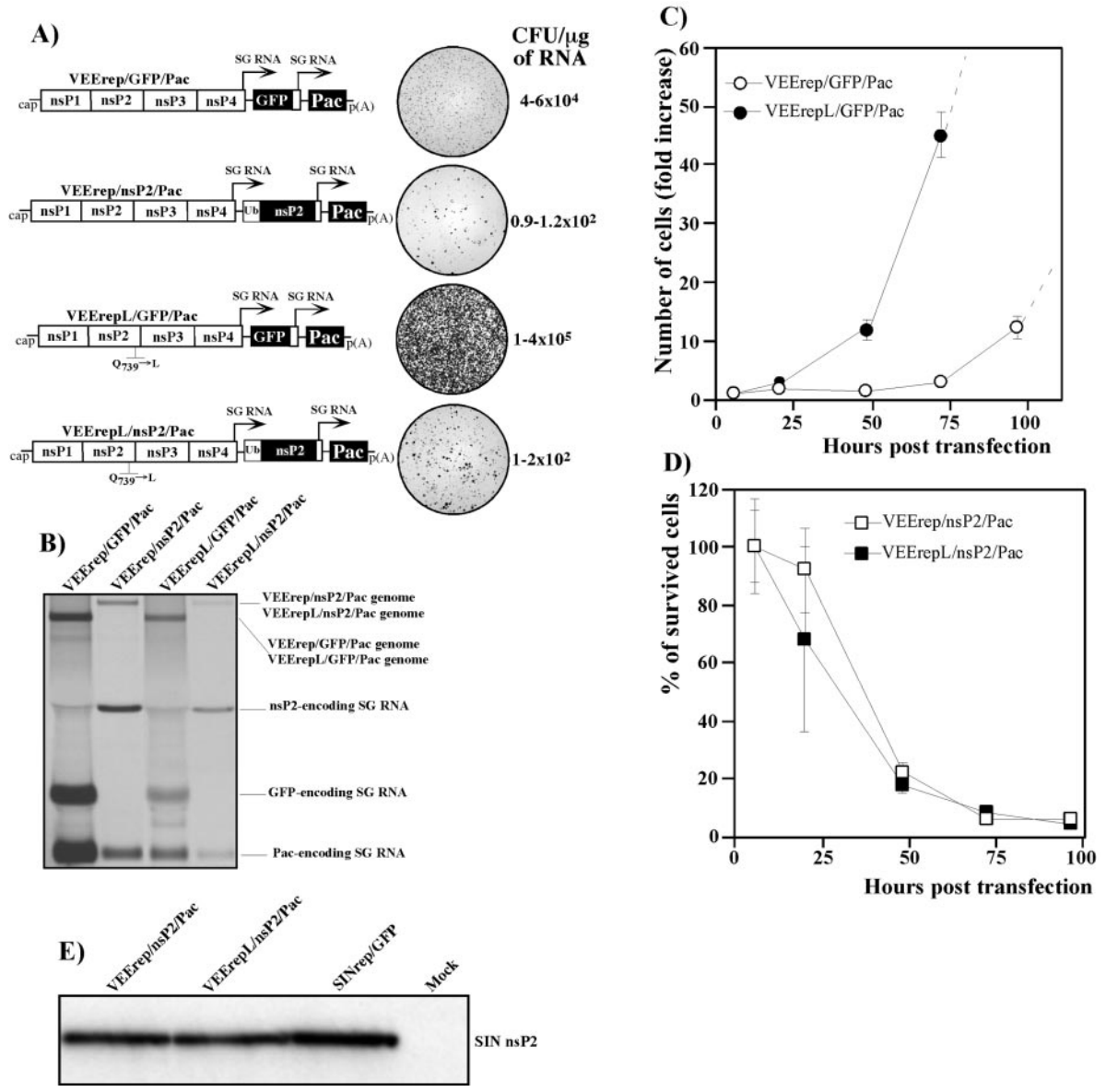

FIG. 1. Analysis of the effect of SIN nsP2 expression on cell survival. (A) Schematic representation of VEE replicons expressing SIN nsP2 and analysis of their ability to establish persistent replication. Arrows indicate the positions of the subgenomic promoters. Ub indicates an ubiquitine sequence fused in frame with the SIN nsP2 gene. Pac indicates the puromycin acetyltransferase gene. Different dilutions of the electroporated cells were seeded into 100-mm tissue culture dishes. Puromycin selection was performed as described in Materials and Methods. Pur^r cell colonies were stained with crystal violet at days 4 to 9 posttransfection, depending on their growth rates. The results are presented in CFU per μg of RNA used for transfection. The ranges indicate variations between the experiments. (B) Analysis of replicon genome RNA replication and transcription of the subgenomic RNAs. The details of the experiments are described in Materials and Methods. RNA labeling was performed with [^{3}H]uridine between 15 and 20 h postelectroporation. The positions of genomic and subgenomic RNAs are indicated. (C and D) Analysis of growth of the cells transfected with VEE replicons expressing GFP (C), and analysis of cell survival at different times posttransfection with VEE replicons expressing wt SIN nsP2 (D). Equal numbers of cells were seeded into six-well Costar plates (one-tenth of the electroporated cells per well). Puromycin selection (10 μg/ml) was performed between 6 and 48 h posttransfection. The cells were then incubated in puromycin-free medium, and viable cells were counted at the indicated times. The data were normalized on the number of viable adherent cells determined at 6 h posttransfection. Panels C and D present the results of the same experiment. The data were separated between two panels for clarity of presentation, because the cells containing GFP-expressing replicons were capable of efficient growth, and cells with VEE replicons expressing wt SIN nsP2 were dying. One of the multiple reproducible experiments is presented. The standard deviations are indicated. (E) Accumulation of SIN nsP2 in the BHK-21 cells transfected with VEE replicons expressing wt SIN nsP2 and with SINrep/GFP replicon (8). Cell lysates were prepared at 20 h posttransfection and analyzed by Western blotting as described in Materials and Methods.

Venezuelan equine encephalitis virus (VEE) genome. The VEE genome-based, self-replicating RNAs (replicons), which encode no structural genes, are less cytopathic than are similar SIN-based constructs. These replicons were shown to be capable of establishing a persistent replication in mammalian cells without a strong alteration in cell growth (28). The VEE replicons used in the present study differed by one amino acid in the nonstructural protein nsP2 (Fig. 1A). One of them, namely,

the wt replicon VEErep, encoding wt nsPs, replicated efficiently in the BHK-21 cells upon delivery by electroporation and caused a detectable level of CPE. However, after the first 2 days of efficient replication (the acute phase), the replicon was able to establish persistent replication in ~10% of the initially transfected cells (28). The second replicon, VEErepL, differed from the VEErep only by a single point mutation in nsP2 (Q_{739}→L) and that additionally strongly decreased its ability to cause CPE. The latter replicon demonstrated a lower level of replication, and this, in turn, led to the survival of essentially all of the transfected BHK-21 cells (28). These two VEE genome-based expression systems were an efficient means of expressing SIN nsP2 in all of the cells used in the experiments and for generating quantitative data (see below). Due to different replication efficiencies, replicons were expected to express at different levels and/or different rates heterologous proteins, which are of interest to us (e.g., nsP2 in the present study).

The expression cassettes were designed as double subgenomic constructs (Fig. 1A), and one of the subgenomic promoters controlled Pac expression. The expression of this protein allowed us to eliminate the background of untransfected cells that could be present in the cell population after transfection and, later, to prevent the accumulation of cells that had cleared the replicons. The second subgenomic promoter drove the expression of SIN nsP2 or GFP (in the control constructs). To produce the protein that starts from the natural, cleavage-generated, N-terminal amino acid, alanine, nsP2 was cloned as a fusion with a ubiquitin-coding sequence.

The in vitro-synthesized replicon RNAs were transfected into BHK-21 cells. Different numbers of the transfected cells were then plated into tissue culture dishes, and the replicon-containing cells were selected in the presence of puromycin starting from 6 h posttransfection. In preliminary experiments, this time was found to be sufficient for replicons to establish replication and make cells resistant to puromycin. Colonies of Pur^r cells were stained at 4 to 9 days posttransfection.

The control replicons VEErep/GFP/Pac and VEErepL/GFP/Pac expressing Pac and GFP genes established persistent replication very efficiently, and formed 4×10^4 to 6×10^4 and 1×10^5 to 4×10^5 colonies of Pur^r cells, respectively, per μg of transfected replicon RNA. The expression of SIN nsP2 caused a strong decrease in the replicons' ability to persistently replicate. VEErep/nsP2/Pac and VEErepL/nsP2/Pac replicons developed 0.9×10^2 to 1.2×10^2 and 1×10^2 to 2×10^2 Pur^r colonies per μg of transfected RNA, respectively.

The lower colony-forming activity of the replicons could be explained either (i) by the direct negative effect of SIN nsP2 on cell physiology; (ii) by transcomplementation of VEE replicative complexes with SIN nsP2, leading to an increase in RNA replication and overproduction of viral nsPs; or (iii) by the interference of SIN nsP2 with the replication of the VEE-specific RNAs, leading to decreased viral RNA synthesis and, thus, lower Pac synthesis that could, in turn, make cells sensitive to translation arrest caused by the puromycin.

To distinguish between these possibilities, virus-specific RNAs were metabolically pulse-labeled with [^{3}H]uridine in the presence of ActD (Fig. 1B). We found that VEErep-based constructs had higher levels of replication than those of the VEErepL-based analogs and, importantly, that expression of the wt SIN nsP2 did not increase RNA replication and transcription. In contrast, the expression of this protein had some negative impact on the synthesis of replicon-specific RNAs. Thus, the lower number of surviving, Pur^r, replicon-containing cells that were capable of forming colonies was not a result of more efficient RNA replication.

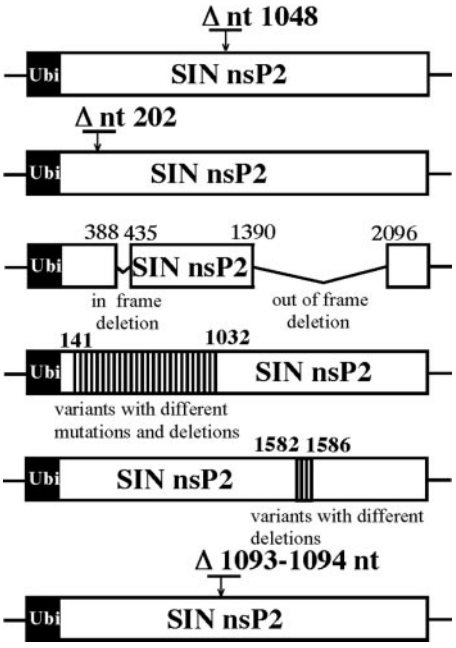

FIG. 2. Accumulation of mutations in wt SIN nsP2 encoded by VEE replicons. Pur^r cell colonies formed after transfection of VEErep/nsP2/Pac and VEErepL/nsP2/Pac replicons were randomly selected, and the genome fragment encoding SIN nsP2 was sequenced. Replicons in some of the clones expressed multiple variants of the SIN nsP2-coding gene.

In parallel experiments, after 48 h of drug treatment of transfected cells, the medium was replaced with a medium lacking puromycin. This time period was found to be sufficient to eliminate all of the untransfected, Pur^s cells. In the absence of puromycin, cells transfected with VEErep/GFP/Pac and VEErepL/GFP/Pac sustained their growth (Fig. 1C), but VEErep/nsP2/Pac- and VEErepL/nsP2/Pac-transfected cells continued to die, with very few colonies remaining (Fig. 1D). These very low numbers of surviving cells indicated that the inefficient colony formation by the cells containing VEE replicons expressing wt SIN nsP2 was not a result of downregulation of RNA replication (a few days posttransfection) below the level required for supporting the Pur^r phenotype. To rule out the possibility that the cytopathic phenotype is a result of a very high level of wt SIN nsP2 expression by VEE replicons, its accumulation level was compared to that in the cells transfected with SIN replicon SINrep/GFP (8). The results presented in Fig. 1E indicate that by 20 h posttransfection, VEE replicons expressed wt SIN nsP2 to a level similar to that found in the SINrep/GFP-containing cells.

VEErep/nsP2/Pac and VEErepL/nsP2/Pac demonstrated a very cytopathic phenotype; however, a small fraction of the transfected cell was capable of growth and formed Pur^r foci. To further understand the reason for cell survival, we randomly

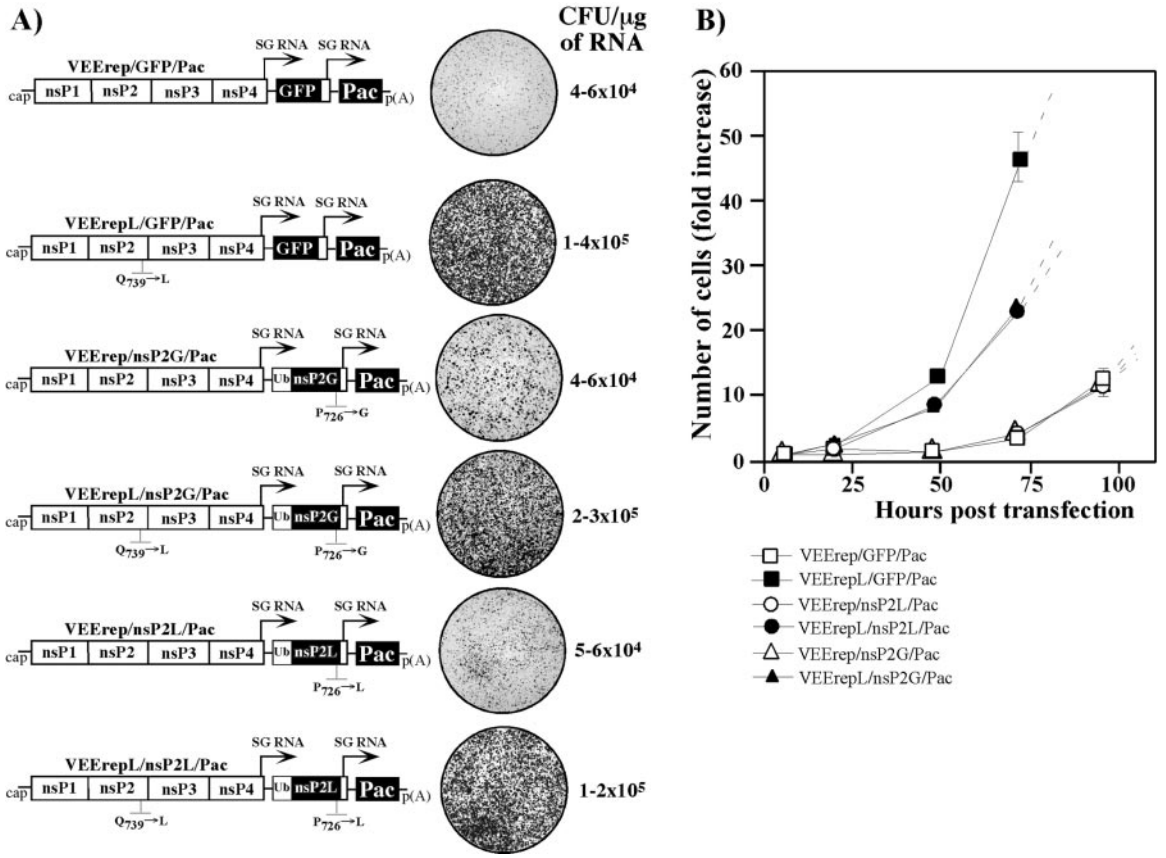

FIG. 3. Analysis of the effect of mutated SIN nsP2 on cytotoxicity of the VEE replicons. (A) Schematic representation of VEE genome-based replicons expressing SIN nsP2 containing the previously identified adaptive mutations (9) and analysis of their ability to establish persistent replication and develop Pur^r foci. Arrows indicate the positions of the subgenomic promoters. Ub indicates a ubiquitine sequence fused in frame with the SIN nsP2 gene. (B) Analysis of growth of the cells carrying VEE replicons expressing SIN nsP2 with P_{726}→G and P_{726}→L mutations. The details of the experimental procedure are described in Materials and Methods.

selected six colonies containing VEErep/nsP2/Pac or VEErepL/nsP2/Pac replicons. The entire SIN nsP2-coding subgenomic RNAs were sequenced, and, in all cases, we found the mutations that destroyed the open reading frame (Fig. 2). The nsP2-coding sequence accumulated either short or extended out-of-frame deletions that prevented the translation of at least one-third of the protein. Thus, the inability of VEE replicons to express an entire wt SIN nsP2 made them noncytopathic and capable of persistent replication in BHK-21 cells. Taken together, the data indicated that the expression of wt SIN nsP2 strongly affects cell biology and might lead to cell death.

Adaptive mutations in SIN nsP2 reduce its cytotoxicity. In our previous studies, we selected a number of SIN replicons that demonstrated lower levels of cytotoxicity. These replicons contained adaptive mutations in the carboxy-terminal part of nsP2, in the sequence located outside of the helicase and protease domains, previously identified in this protein (1, 9). To further understand the role of two mutations in the 726 position of the SIN nsP2, we cloned these mutated genes into VEE replicons under the control of a subgenomic promoter (Fig. 3A). The adaptive mutations P_{726}→L and P_{726}→G in SIN nsP2 blocked the cytotoxicity of this protein in the context of either VEErep and VEErepL replicons. SIN nsP2L- and SIN nsP2G-expressing replicons demonstrated the same abilities to form Pur^r colonies as did the original vectors expressing GFP- and Pac-coding subgenomic RNAs (Fig. 3A), and replicon-containing cells demonstrated an efficient growth (Fig. 3B). These data indicated that the previously detected effect of mutations in SIN nsP2 on viral and replicon cytotoxicity (7, 9, 27) was likely a result of changes in the function(s) of this protein in virus-host cell interactions, and the carboxy-terminal part of nsP2 appears to play a critical role in the ability of this protein to cause CPE.

SIN nsP2 with mutated protease domain remains cytotoxic. The simplest hypothesis that might explain the cytotoxic effect of nsP2 was that it resulted from the nsP2-associated protease activity processing cellular protein(s) that play critical roles in cell biology. The adaptive mutations, found in the carboxy-terminal fragment of nsP2, could potentially affect the specificity of the protease and/or its activity. To test this possibility, we mutated Cys_{481} in SIN nsP2 expressed by the VEE replicons to Ser (Fig. 4A). Based on previously published data (36) and our unpublished results, this mutation completely abolished nsP2 protease activity. However, cells transfected with these VEE replicons, VEErep/nsP2m/Pac and VEErepL/nsP2m/Pac, formed Pur^r colonies 100-fold less efficiently than did the cells transfected with VEErep/GFP/Pac and VEErepL/GFP/Pac vectors (Fig. 4A), at levels similar to those deter-

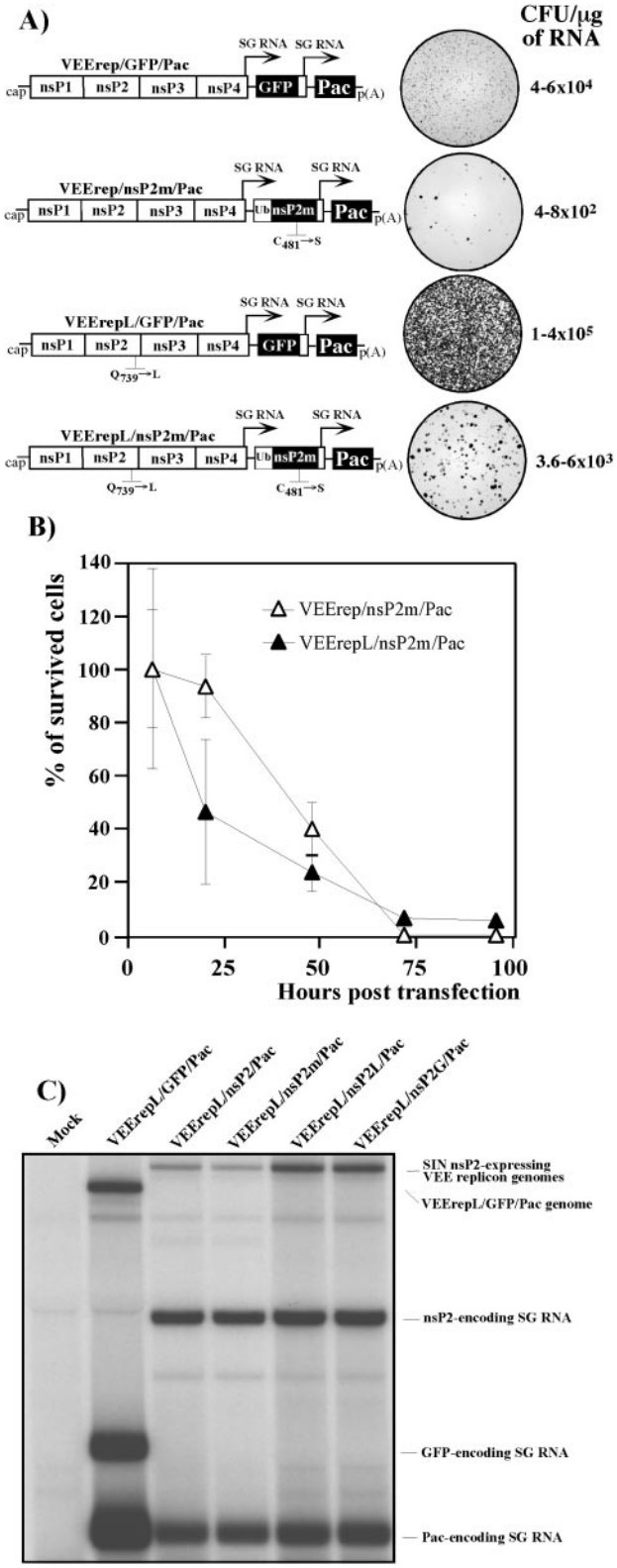

FIG. 4. Analysis of the cytotoxicity of SIN nsP2 with mutated protease. (A) Schematic representation of VEE genome-based replicons expressing SIN nsP2 containing C_{481}→S mutation and analysis of their ability to establish persistent replication and develop Pur^r foci. (B) Survival of the cells transfected with VEE replicons expressing SIN nsP2 with C_{481}→S mutation. The data were normalized on the number of viable adherent cells determined at 6 h posttransfection. (C) Analysis of replicons' genome replication and transcription of the subgenomic RNAs. RNA labeling was performed with [^{32}P]phosphoric acid. The details of the experiments are described in Materials and Methods.

mined for VEE replicons expressing wt SIN nsP2 (see Fig. 1A). As did VEErep/nsP2/Pac and VEErepL/nsP2/Pac, the nsP2m-expressing replicons killed almost all of the cells within 3 days posttransfection (Fig. 4B), which was indicative of the cytotoxicity of the wt SIN nsP2 as being either protease independent, or determined by the synergistic effect of proteolytic activity and another, additional function of this protein that is not characterized yet. The latter possibility is supported by the fact that VEErep/nsP2m/Pac and VEErepL/nsP2m/Pac replicons formed 4-to 30-fold more Pur^r cell colonies than did their counterparts VEErep/nsP2/Pac and VEErepL/nsP2/Pac, respectively. Interestingly, SIN nsP2 with mutated protease downregulated the replication of VEE replicons to the same level as did wt SIN nsP2 (but not nsP2L or nsP2G), suggesting that interference with VEE RNA replication did not depend on protease activity of SIN nsP2 (Fig. 4C).

The detected strong differences in the cytotoxicity of different replicons did not result from changes in the stability of SIN nsP2. The mutated and wt proteins were found in the cells in essentially the same concentrations (Fig. 5A), regardless of the VEE replicons used for expression. The mutations also did not affect the intracellular distribution of SIN nsP2 (Fig. 5B), which was observed in the cytoplasm and nuclei of the transfected cells.

SIN nsP2 protein downregulates transcription of cellular RNAs. The data from our previous studies suggested that SIN nsP2 plays a critical role in virus-host cell interactions (12, 15), and the adaptive mutations in this protein have a strong effect on the ability of SIN virus and SIN replicons to inhibit transcription (15). Thus, we tested whether the downregulation of cellular transcription was a direct function of nsP2. The in vitro-synthesized RNAs of GFP-expressing VEE replicons and replicons encoding (i) wt SIN nsP2, (ii) nsP2 with adaptive mutations, and (iii) nsP2 with mutated protease were electroporated into BHK-21 cells. RNAs were pulse-labeled with [^{3}H]uridine at 15 to 20 h posttransfection in the absence of ActD. The labeling time was chosen based on the fact that SIN infection caused its main effect on cellular transcription by 16 h postinfection (15). We detected profound differences in the ability of replicons to downregulate transcription (Fig. 6A). Both VEErep and VEErepL replicons expressing either wt SIN nsP2 or nsP2 lacking protease activity induced a 10-fold decrease in the transcription of cellular RNAs. SIN nsP2 with adaptive P_{726}→G and P_{726}→L mutations in the carboxy terminus, expressed in the context of wt VEErep, downregulated transcription by 2.5-fold, and the same mutated proteins expressed by the VEErepL mutant did not affect transcription more than did vectors encoding GFP in the subgenomic RNA. Thus, SIN nsP2 itself served as a strong inhibitor of cellular transcription.

The inhibition of transcription correlated with the development of both CPE (see above) and apoptosis (Fig. 6B). By using anti-phospho-histone H2A.X antibodies, we readily de-

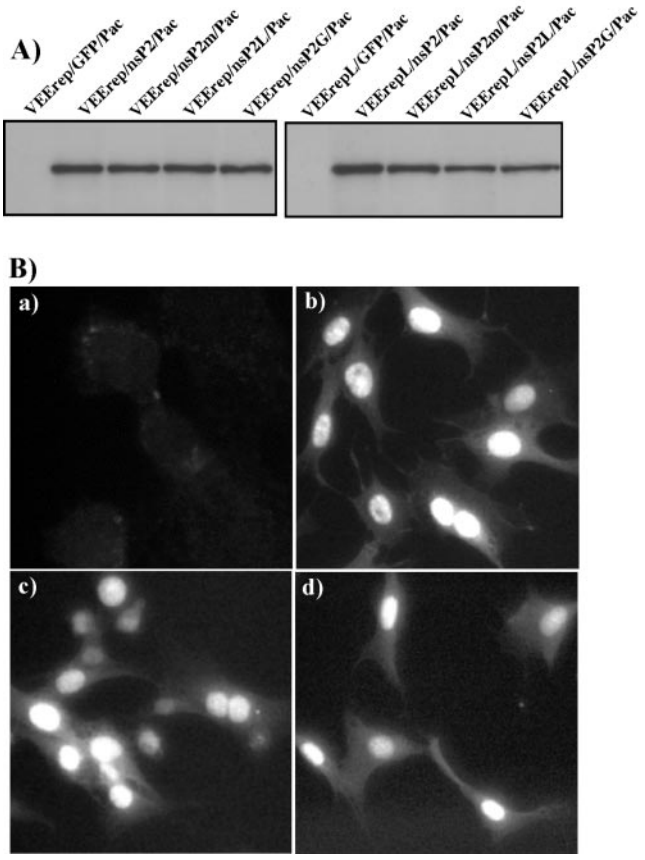

FIG. 5. Accumulation of SIN nsP2 in the BHK-21 cells transfected with VEE replicons expressing different forms of SIN nsP2. (A) Cell lysates were prepared at 20 h posttransfection and analyzed by Western blotting as described in Materials and Methods. (B) For analysis of nsP2 distribution, BHK-21 cells were electroporated by in vitro-synthesized RNAs and, at 16 h posttransfection, stained with rabbit anti-SIN nsP2 and goat anti-rabbit IgG Alexa Fluor 546-labeled secondary antibodies (Molecular Probes). Cells: a, VEErepL/GFP/Pac-transfected cells; b, VEErepL/nsP2/Pac-transfected cells; c, VEErepL/nsP2L/Pac-transfected cells; d, VEErepL/nsP2m/Pac-transfected cells.

tected DNA cleavage in the cells transfected with VEErepL replicons expressing wt SIN nsP2 or nsP2 with mutated protease (Fig. 6Bd and j, respectively). Neither the GFP-expressing replicons nor the replicons expressing SIN nsP2 with adaptive mutations were capable of causing apoptosis. It should be noted that the anti-phospho-histone H2A.X antibodies used in these experiments were capable of detecting very early stages of apoptosis. However, we never detected cellular DNA degradation, which is characteristic of the late stages of apoptosis, even in the already detached cells containing SIN nsP2-expressing replicons (data not shown).

Effect of nsP2 expression from DNA cassettes. The strong effects of wt SIN nsP2 on cell viability and apoptosis development detected in the above-described experiments could potentially represent a synergistic effect of the functioning of this protein, replication of VEE-specific RNAs, and accumulation of VEE-specific nsPs. To rule out the possibility of the complex effect, we designed a variety of cassettes expressing different forms of nsP2 from strong chicken β-actin promoter (Fig. 7A). The cassettes expressed wt SIN nsP2, nsP2 with adaptive mutation P_{726}→L and nsP2 with mutated protease (C_{481}→S). We were also interested in testing whether SIN nsP2, when expressed in the context of uncleaved or cleavage-competent P123, could interfere with cellular transcription. Such long sequences probably could not be expressed from VEE replicons and, therefore, we cloned them under the control of a β-actin promoter. The p12m34 and p1234 cassettes differed, since the former carried a C_{481}→S mutation in nsP2 and an Ubi sequence in the junction of nsP3 and nsP4 to promote cleavage of P1234 in the context of mutated nsP2 protease. From our other studies, we knew that the insertion of an Ubi sequence into the nsP3/nsP4 junction of the SIN genome would not affect viral replication (data not shown). Both cassettes also contained a GFP insertion in the nsP3-coding sequence to monitor the expression level of nsPs. The GFP insertion into nsP3 also caused no effect on RC formation and function (11). The p1*2*34 cassette contained previously described mutations at the cleavage sites nsP1/2 and nsP2/3, making the processing of P123 impossible (35), and an Ubi sequence between nsP3 and nsP4 to promote cleavage of the latter protein. Thus, p1234 generated SIN P123 capable of further complete processing, and p12m34 and p1*2*34 expressed uncleaved P123 (fused with Ubi) and nsP4.

All of the designed cassettes were cotransfected with a Pac-expressing plasmid into BHK-21 cells (see Materials and Methods for details). At 24 h posttransfection, puromycin selection was applied for 24 h to eliminate the background of untransfected cells, and cell growth was analyzed by counting the cells at the indicated time points (Fig. 7B and C). Expression of the wt SIN nsP2 and C_{481}→S mutant was toxic for the cells, and essentially all of the transfected cells were dead within 3 days posttransfection, which was similar to the findings presented above. In contrast, the nsP2 with P_{726}→L mutation was incapable of causing cell death. However, cells transfected with the plasmids encoding either authentic or mutated nsP2 produced these proteins at very high concentrations (data not shown), and even the P_{726}→L mutant protein caused detectable CPE within the first 2 days posttransfection, and then cells were able to resume their growth.

Upon transfection, the p1234 cassette was as cytotoxic as p2 (expressing nsP2 alone), but the point mutation C_{481}→S in p12m34 completely abolished the ability of expressed proteins to cause CPE. Essentially the same result was achieved when protease-active nsP2 was expressed as a P123 precursor with mutated cleavage sites in the p1*2*34 cassette. Taken together, these data indicate that cytotoxicity and, most likely, the ability to downregulate cellular transcription are functions of SIN nsP2 present only in a free form but not in the context of a polyprotein precursor.

DISCUSSION

In the infected cells, SIN expresses only four nonstructural proteins, namely, nsP1, nsP2, nsP3, and nsP4, which form, together with host factors, replicative enzyme complexes functioning in the synthesis of virus-specific RNAs (38). However, SIN replication leads to a strong modification of the intracellular environment to meet the needs of efficient viral replication. At the same time, as shown for some other viral infections (2, 4, 24, 40), SIN replication appears to interfere with a cel-

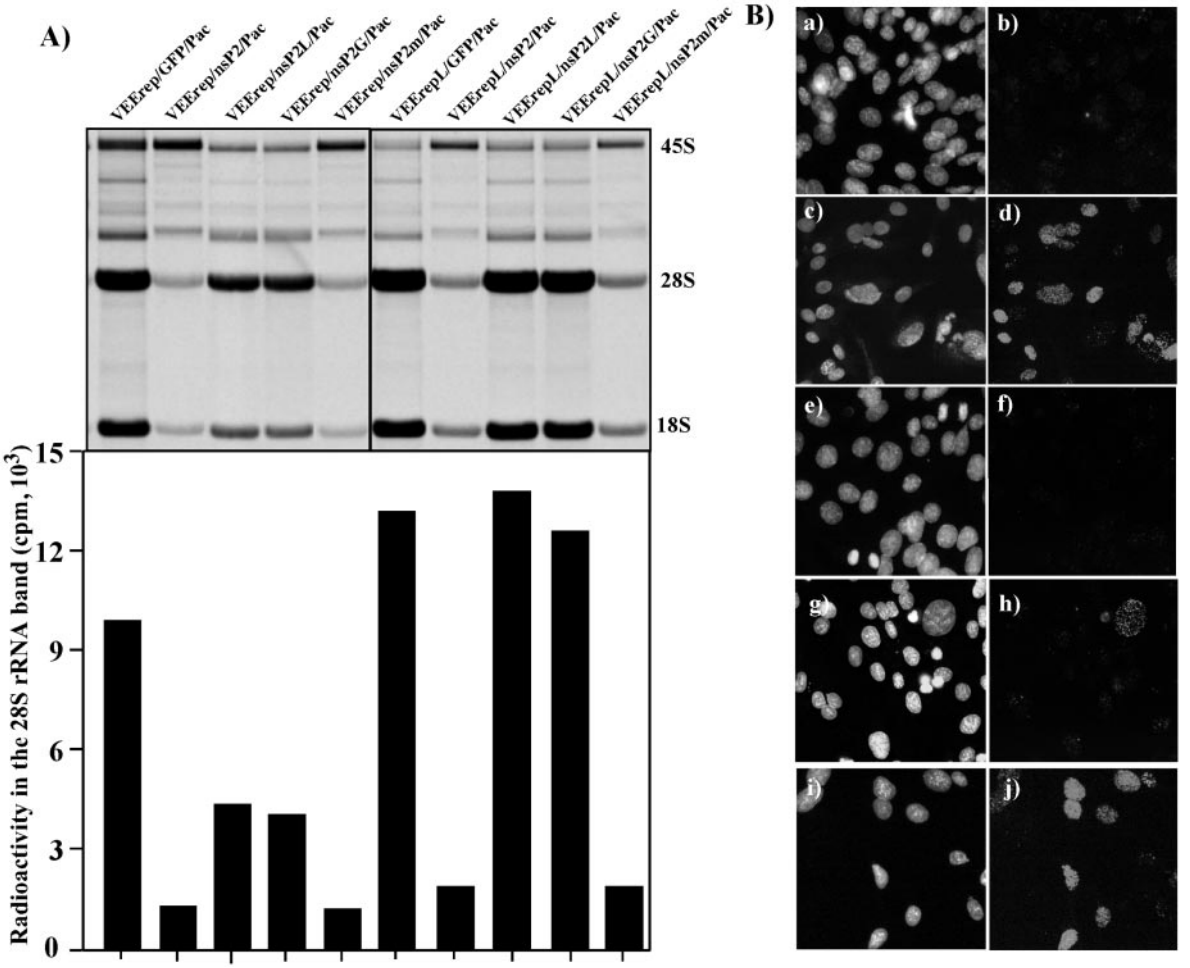

FIG. 6. Inhibition of transcription and development of apoptosis in the BHK-21 cells transfected with VEE replicons expressing different forms of SIN nsP2. (A) BHK-21 cells were electroporated by 5 μg of the in vitro-synthesized RNAs. At 15 h posttransfection, cellular RNAs were labeled with [^{3}H]uridine for 5 h and analyzed by RNA gel electrophoresis under the conditions described in Materials and Methods. For quantitative analysis, the rRNA bands were excised from the PPO-impregnated gels (shown in the upper panel), and the radioactivity was measured by liquid scintillation counting (lower panel). One of two reproducible experiments is presented. (B) For detection of apoptosis, BHK-21 cells were electroporated by in vitro-synthesized RNAs and, at 48 h posttransfection, stained with mouse anti-phospho-histone H2A.X antibodies and goat anti-mouse IgG Alexa Fluor 546-labeled secondary antibodies (Molecular Probes) as described in Materials and Methods in subpanels b, d, f, h, and j. DAPI staining of the same cells is shown in subpanels a, c, e, g, and i. Cells: a and b, VEErepL/GFP/Pac-transfected cells; c and d, VEErepL/nsP2/Pac-transfected cells; e and f, VEErepL/nsP2L/Pac-transfected cells; g and h, VEErepL/nsP2G/Pac-transfected cells; i and j, VEErepL/nsP2m/Pac-transfected cells.

lular antiviral response and downregulates cell signaling aimed at activation of the antiviral state in the uninfected cells, making them resistant to successive rounds of infection (12). Thus, it is reasonable to expect that SIN nsPs are involved not only in the replication of the viral genome and transcription of the subgenomic RNA but also in other processes leading to the downregulation of the antiviral response. SIN infection was shown to inhibit cellular transcription and translation, using this shutoff as an efficient means to interfere with the development of a cellular reaction (15). Our studies (11) and results from other research groups (13, 25) demonstrated that, in the infected cells, one of the nonstructural proteins, nsP3, forms high-order complexes attached to stable cellular structures. Thus, it is unlikely that nsP3 affects cellular transcription and translation. Another SIN protein, nsP4, which expresses RNA-dependent RNA polymerase activity, is present at a very low concentration in the cells and is apparently not involved in the alteration of cellular macromolecular synthesis as well. Of the two other nsPs, nsP2 is a better candidate as a critical factor in virus-host cell interactions. In contrast to nsP1, nsP2 is not predicted to bind to cellular membranes, and it was not only found in association with RCs but was also detected in the cytoplasm (15). Moreover, a large fraction of nsP2 was found in the nuclei (15, 26). Most importantly, mutations in this protein were shown to have a strong impact on the development of transcriptional and translational shutoffs occurring in mammalian cells during SIN replication and, consequently, on the ability of the virus to cause CPE (7, 9, 27). The adaptive mutations leading to less-cytopathic viral or replicon phenotypes emerged in the carboxy-terminal fragment of nsP2 (amino acids 726 and 779) that appears to be not directly associated with the protease and helicase domains described

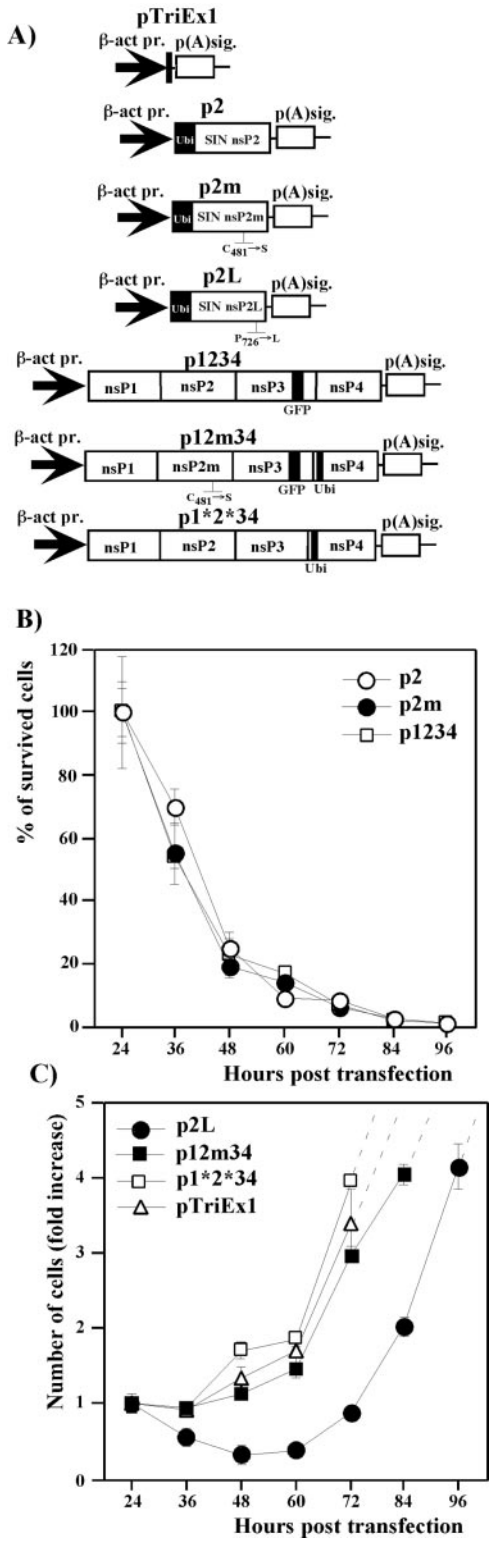

FIG. 7. Analysis of the effect of SIN nsP2 expression from plasmid DNAs on cell survival. (A) Schematic representation of plasmid-based cassettes, containing chicken β-actin promoter and expressing different forms of SIN nsP2. Ubi indicates a ubiquitine sequence fused in frame with SIN nsP2 gene. (B and C) Analysis of the cell survival (B) and cell growth (C) at different times posttransfection with the plasmids. The details of the experimental procedure are given in Materials and Methods. Panels B and C present the results of the same experiment. The data were separated between two panels for clarity of presentation. The data were normalized on the number of viable adherent cells determined at 24 h posttransfection.

for this protein (9, 27, 28). In the present study, we were interested in further understanding the effect of these mutations on SIN nsP2 functioning and tested whether SIN nsP2 is directly involved in the development of CPE. VEE replicons were used for its expression for multiple reasons. In contrast to traditionally used plasmid-based expression cassettes, the in vitro-synthesized replicon RNAs could be transfected in essentially 100% of the cells, where they synchronously initiated the expression of heterologous genes within a few hours (28). In contrast to SIN and SFV, VEE-based replicons demonstrated a low level of RNA replication and had little effect on major cellular processes (28). The wt VEE replicon, VEErep, could readily establish persistent replication (at least in a large fraction of BHK-21 cells), and the replicon with additional adaptive mutations, VEErepL, replicated essentially in each transfected cell without affecting cell growth (28). Based on the ability of the VEErep to cause some level of CPE within the first hours posttransfection, we expected by using these expression constructs to be able to detect even small additional changes in cytotoxicity. Noncytopathic VEErepL mutants were expected to detect strong increases in cytotoxicity caused by the expression of heterologous genes.

The expression of wt SIN nsP2 changed the phenotypes of both replicons to those that were more cytopathic, without increasing the levels of RNA replication, indicating that this was a strong, dominant effect that likely did not depend on the VEE vectors themselves. The cytotoxicity of SIN nsP2 was not determined by its protease activity (or at least not only by its protease activity), and the mutation in the active site of the protease had only a minor effect on the ability of this protein to induce CPE. The introduced adaptive mutations, previously found in the noncytopathic SIN replicons (9), reverted the phenotype of the expression cassettes to the original, noncytopathic one. These mutations did not change the stability of SIN nsP2 and/or compartmentalization of this protein in the cells. However, it should be noted that the expression of nsP2 with P_{726}→L or P_{726}→G mutations from VEE replicons had a detectable negative effect on cell growth (Fig. 3B), and the overexpression of the mutant protein from the β-actin promoter caused the death of some fraction of the cells (Fig. 7C). Nevertheless, the difference between the effects of wt and mutant forms of SIN nsP2 on cell physiology was very strong in all of the experiments performed.

The CPE caused by wt SIN nsP2 was at least, in part, determined by the ability of this protein to inhibit cellular transcription (Fig. 6A). This was a plausible explanation of why the SIN replicons containing the mutations in the carboxy-terminal part of nsP2 adapted to persistent replication in the cultured cells but, importantly, only in the cells with defects in alpha/beta interferon production or signaling (1, 9, 27). Other cell lines were capable of activating an efficient viral stress-induced cell response and stopping the synthesis of replicon-specific RNAs.

Based on the present data, the interference with cellular

transcription appears to be a direct function of nsP2, and this function is not directly associated with protease and helicase activities. The results demonstrated in Fig. 6A also suggest the possibility that the expression of wt SIN nsP2 could affect the processing of at least pre-rRNAs, because the latter RNAs accumulated at a higher concentration in the cells expressing wt nsP2 or C_{481}→S mutant. However, this assumption needs additional experimental support. Interestingly, we did not find any significant direct effect of the expression of wt nsP2 or any of its mutants on cellular translation (data not shown). At 16 h postinfection, cells expressing SIN nsP2 continued to synthesize their proteins as efficiently as did the cells transfected with attenuated VEErepL/GFP/Pac vectors.

Our previous studies demonstrated that changes in the processing of the polyprotein encoding SIN nsPs had a strong effect on the development of transcriptional shutoff in the infected cells, suggesting that only fully processed nsP2 is functional in the regulation of cellular transcription (15). To further understand this phenomenon, we expressed SIN ns polyprotein, containing different mutations affecting processing, in a context different from that provided by the VEE replicons. In agreement with VEE replicon-based experiments, expression of wt SIN nsP2 or C_{481}→S mutant protein from a strong chicken β-actin promoter caused CPE. The P_{726}→L mutation made this protein noncytotoxic. Thus, both of the expression systems (VEE genome- and plasmid-based) generated consistent data. The expression of P1234, which is competent of complete processing to individual nsPs from the pTriEx1 vector, was also cytopathic. However, alterations in the P123 processing, by making mutations in the active center of protease or in the cleavage sites, inactivated the ability of SIN nsP2 to cause cell death. Most likely, this was a result of the inability of nsP2 to inhibit cellular transcription because of the different compartmentalization and/or conformation of this protein (15).

Inhibition of cellular transcription appears to be a mechanism used by a variety of viruses. This phenomenon was previously described and studied in detail for poliovirus and vesicular stomatitis virus infections (41, 44, 45). The NSs protein of Rift Valley fever virus was also found to be a critical factor for development of transcriptional shutoff (3, 6), and we can expect that the number of examples will increase in the future. However, the virus-dependent inhibition of cellular transcription appears to be not a universal event, even among the alphaviruses. Our previous studies strongly suggest that at least VEE- and EEE-based replicons are incapable of inhibiting transcription as efficiently as does SIN (28). One of the interesting questions that came out of the present study is what is the difference between the functional activities of SIN nsP2 and nsP2s derived from different alphaviruses? In contrast to SIN, replication of VEE- and EEE-based replicons does not cause profound changes in cellular biology. These replicons do not inhibit cellular transcription and translation to levels that make it impossible for cells to survive and further proliferate and, thus, these replicons readily establish persistent replication (28). Based on the data presented in Fig. 6A, the replication of VEE-specific, wt virus genome-based RNAs induces a detectable decrease in cellular transcription (that is additionally induced by expression of SIN nsP2 even with P_{726}→G or L mutations) during the acute replication phase. These changes stop cell division for at least 48 h (compare the growth of the cells transfected with VEErep and VEErepL constructs in Fig. 1C and 3B) and, during this time, replicons induce a wide variety of cellular genes comprising the virus-induced cell response (unpublished data), leading to the release of a variety of cytokines. We speculate that, in contrast to SIN, the replication of VEE and VEE-based replicons is strongly resistant to a virus-induced cell response and, first of all, to the autocrine and paracrine action of interferon and, therefore, this virus uses a different means of interfering with the developing antiviral reaction. However, this VEE-specific mechanism requires further investigation.

In conclusion, we demonstrated that (i) SIN nsP2 is critically involved in CPE development, not only during the replication of SIN-specific RNAs, but also when this protein is expressed alone from different expression cassettes. (ii) The cytotoxic effect of SIN nsP2 appears to be at least partially determined by its ability to cause transcriptional shutoff. (iii) These functions of SIN nsP2 are determined by the integrity of the carboxy-terminal peptide of this protein located outside its helicase and protease domains rather than by its protease activity. (iv) The cytotoxic activity of SIN nsP2 strongly depends on the presence of this protein in a free form. Alterations in P123 processing abolish the ability of nsP2 to cause CPE.

ACKNOWLEDGMENTS

We thank Peter Mason and Mardelle Susman, technical editor, for critical reading and editing of the manuscript.

This study was supported by Public Health Service grant AI050537.

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